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Award Number: DAMD17-02-1-0651

TITLE: Signaling Pathways Controlling the Growth and Proliferation of Drosophilae Perineurial Glial Cells

PRINCIPAL INVESTIGATOR: Michael Stern, Ph.D.

CONTRACTING ORGANIZATION: Rice University
Houston, Texas 77251-1892

REPORT DATE: May 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2003	Annual (1 May 02 - 30 Apr 03)

4. TITLE AND SUBTITLE
Signaling Pathways Controlling the Growth and Proliferation of Drosophilae Perineurial Glial Cells

5. FUNDING NUMBERS
DAMD17-02-1-0651

6. AUTHOR(S)
Michael Stern, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Rice University
Houston, Texas 77251-1892

E-Mail: stern@rice.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The long term goals of this research are to understand the mechanisms by which *NF1* and its partners control growth using the Drosophila peripheral nerve. This system is advantageous because we can apply a number of powerful molecular genetic methodologies that are not available in other systems. The aims of this project address three specific aspects of growth control. First, we asked if *NF1* acts downstream of a G protein to exert its effects. Based on initial experiments from this grant and related experiments from a different grant, we obtained evidence that *NF1* has dual, opposing roles in glial growth control. Thus, our original hypothesis is incomplete and must be remodeled. Second, we tested further the hypothesis that motor neuron activity affects perineurial glial growth. So far, our experiments on triple mutants exhibiting extremely elevated neurotransmitter signalling failed to find significant effects on glial growth. Further experiments are in progress. Third, we asked if perineurial glial growth can be genetically uncoupled from perineurial glial proliferation. We found that expression of a constitutively active Ras does, in fact, cell nonautonomously increase perineurial glial nuclei number, but this increase is not required for the growth effects observed in chromosomal mutants.

14. SUBJECT TERMS
molecular genetics; neuroscience; cell biology; cell signaling; model system genetics

15. NUMBER OF PAGES
42

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

Over the last several years, my lab has been developing the Drosophila peripheral nerve as a system with which to identify and study the signalling pathways controlling growth of the perineurial (outer) glial layer. The idea behind this approach is to apply the various molecular genetic methodologies uniquely available in Drosophila to enable us ultimately to identify all of the relevant genes that interact with *NF1* to control growth, and place *NF1* and these partner genes in as complete a mechanistic context as possible. Then this mechanism could be tested and refined in systems more similar to humans but more difficult to work with (i.e. the mouse). Because all of the experimentation is performed on the acutely dissected third instar larva, there are no complications or caveats associated with experimentation on cell culture systems, and we assay the entire nerve cross section as it exists within the whole organism. We thought that a more complete mechanistic understanding of growth control within peripheral nerves would greatly facilitate the ability to design drugs able to combat neurofibromas. Within this larger context, the specific research being performed under this grant was designed to test particular hypotheses that would increase this mechanistic understanding. The first task was designed to test the hypothesis that the *amnesiac*-encoded neuropeptide acts upstream, and Neurofibromin acts downstream, of a G protein subunit. The second task proposed additional experiments to test the hypothesis that perineurial glial growth is regulated by neurotransmitter release from motor neurons. The third aim was designed to test the possibility that growth and mitosis could be mechanistically uncoupled. Successful completion of these aims would provide important information concerning the control of growth within peripheral nerves at the molecular level.

BODY

Task one: Does Neurofibromin act downstream of a G protein to control perineurial glial growth? First we tested if expression of a constitutively active $G_{\alpha}s$ (called $G_{\alpha}s$*) subunit specifically within peripheral glia could suppress the glial growth phenotype of amn^{X8} and Axs^{R1} mutants. The outcome was negative: expression of this constitutively active G protein was not able to suppress the phenotypes of amn^{X8} and Axs^{R1} mutants. However there were two technical difficulties surrounding the experiments, which make their interpretation problematic, and one conceptual difficulty.

The first technical difficulty was that the viability of amn^{X8} mutants carrying the $G_{\alpha}s$* transgene *UAS-* $G_{\alpha}s$* was unexpectedly low, even in the absence of any *GAL4* element to drive *UAS-* $G_{\alpha}s$* expression. The reason for this low viability is unclear. The second technical difficulty was that the viability of amn^{+} flies expressing *UAS-* $G_{\alpha}s$* with the *gli-GAL4* and *MZ709* elements was also low. We are able to obtain viable larvae by driving *UAS-* $G_{\alpha}s$* with the *MZ317* element and are currently doing so.

The conceptual difficulty is that our work on genes downstream of $G_{\alpha}s$ (*NF1* and protein kinase A), funded by my NIH grant, is calling into question the hypothesis that $G_{\alpha}s$ activity, acting through Neurofibromin and protein kinase A, will reduce perineurial glial growth. In particular, we found that expression of a constitutively active protein kinase A (PKA*) did not suppress the increased perineurial glial growth of amn^{X8} (Jim Yager and Michael Stern, unpublished observation). Furthermore, we found that *NF1* mutations actually suppressed the increased perineurial glial growth conferred by expression of a constitutively active Ras protein (Yager et al., submitted, supplied in

appendix). These results are not possible to explain with the current hypothesis and thus the hypothesis must be modified. Taken together, suggest that *NF1* (and hence, by extension, $G_{\alpha}s$*) has dual, opposing, roles in controlling perineurial glial growth: in this view, *NF1* and $G_{\alpha}s$ activity increase perineurial glial growth via activation of PKA, whereas *NF1* and $G_{\alpha}s$ activity reduce perineurial glial growth via inhibition of Ras. This new hypothesis can easily be tested with slight modifications of the statement of work. Therefore, I recommend that the statement of work be changed to accommodate these new discoveries.

Task two: Further tests of the hypothesis that increased neurotransmitter release from motor neurons (or increased neurotransmitter persistence) affects perineurial glial growth. So far, we have constructed and analyzed two of the six fly lines that we proposed to analyze. These lines are: *eag Sh; NF1* and *eag; ine; NF1*. In preliminary data we found that perineurial glial growth was not significantly affected in each triple mutants compared to the double mutants assayed previously (see Figure 1). Thus, so far we are not able to demonstrate that increased neurotransmitter signalling from the motor neurons activates perineurial glial growth. However, additional data points from these lines, and other lines, must be tested for a believable conclusion.

Figure 1: Effects of multiple neuronal excitability mutations on perineurial glial growth of *NF1* mutants

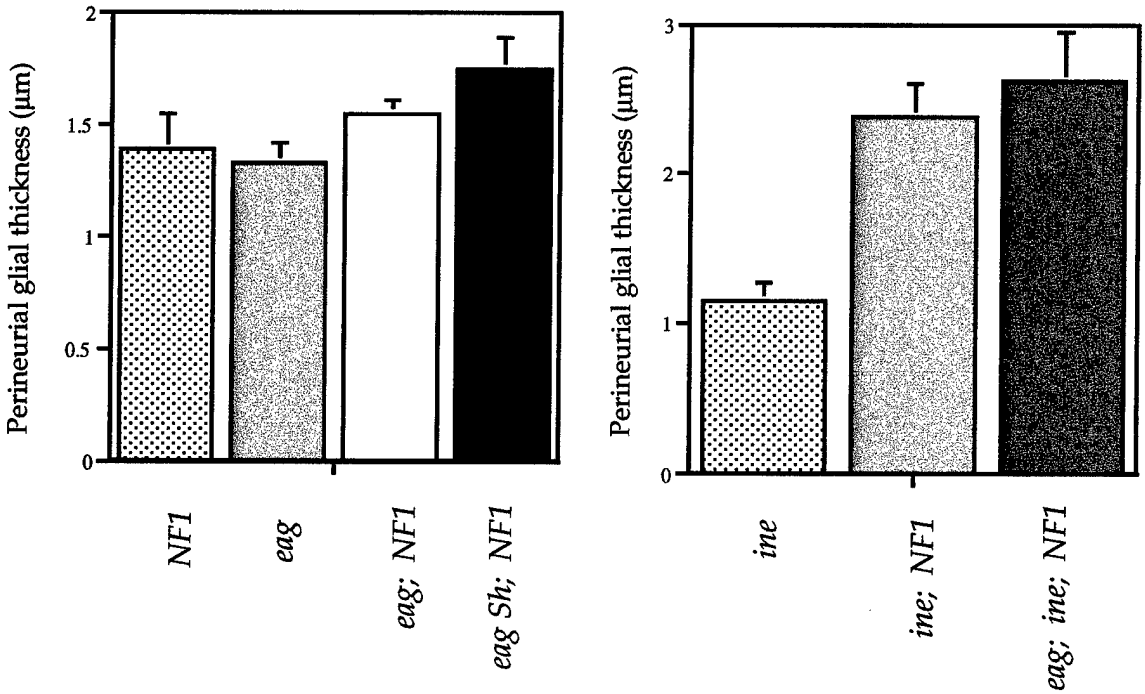

Figure 1: Perineurial glial thickness in mutants of the indicated genotypes. Means +/- SEMs are indicated. Data presented in the left-most three genotypes of the left panel, and the left-most two genotypes of the right panel, were published in Yager et al.

(2001). Data presented for the right-most genotypes of each panel were collected in this study.

Task three: Can perineurial glial growth be genetically uncoupled from perineurial glial proliferation? We counted the number of perineurial glial nuclei per length of nerve from several mutants exhibiting increased glial growth as well as their wildtype controls, as described in the statement of work. We found that the *amn*^{X8} mutant and the *ine; NF1* double mutant exhibited a normal number of perineurial glial nuclei, despite exhibiting significantly increased perineurial glial thickness (Figure 2, also see Figure 4 in Yager et al., submitted). On the surface, this result demonstrates that it is possible to increase glial growth with no significant increase in the number of glial nuclei; thus, one signal sent from the peripheral glia to the perineurial glia is a trophic signal but not a proliferation factor. However, we also observed that larvae expressing the constitutively active Ras^{V12} mutant in peripheral glia exhibited an increase in the number of perineurial glial nuclei, which was significantly greater than in larvae expressing Ras^{+} in peripheral glia (Figure 2, also see Figure 4 in Yager et al., submitted).

Figure 2: Increased number of perineurial glial nuclei in larvae carrying *gli-GAL4* and *UAS-Ras*^{V12}.

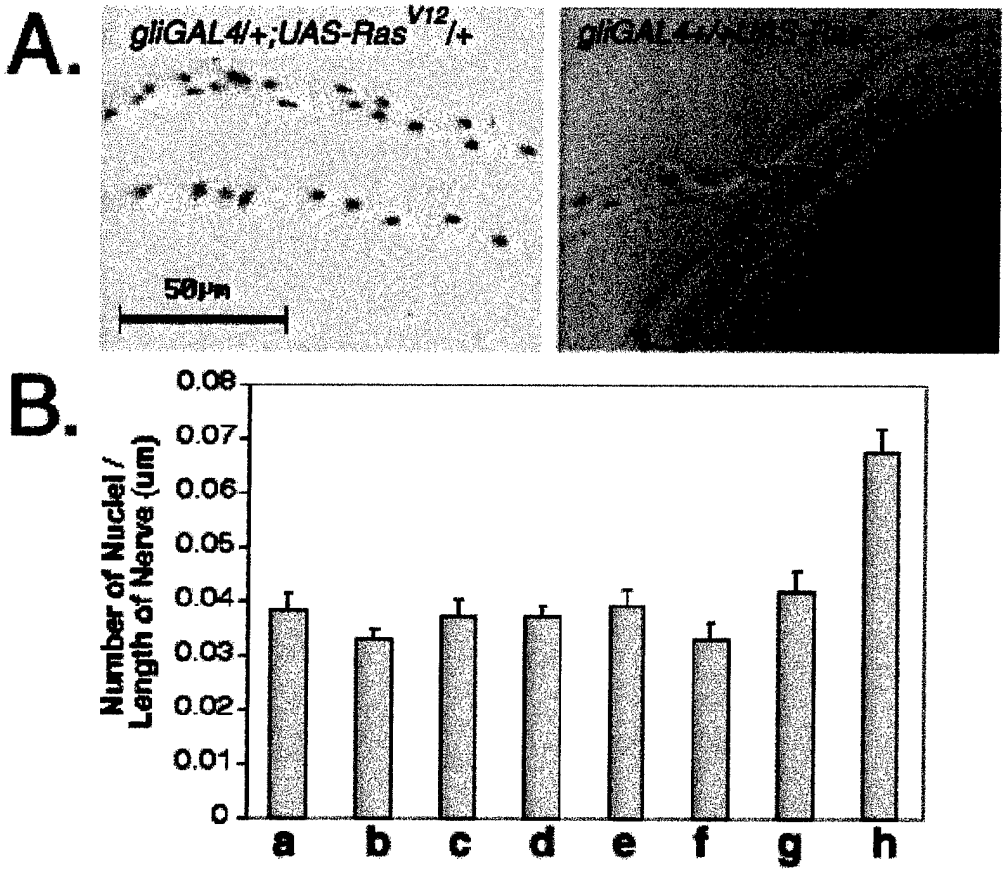

Figure 2: A. Confocal fluorescent images of peripheral nerves from larvae carrying *gli-GAL4* and *UAS-Ras*^{V12} (left panel) and *gli-GAL4* and *UAS-Ras*$^{+}$ (right panel). The large,

nucleus-rich structure on the right of the right panel is the ventral ganglion. B. Means and SEMs of perineurial glial nuclear density from larvae of the following genotypes: a) *K33* (wildtype control for *ine; $NF1^{P2}$*), b) *ine; $NF1^{P2}$*, c) wildtype control for *amn^{X8}*, d) *amn^{X8}*, e) *UAS-Ras^{V12}/+*, f) *gli-GAL4/+*, g) *gli-GAL4 +/+ UAS-Ras^{+}*, h) *gli-GAL4 +/+UAS-Ras^{V12}*. Means were calculated by measuring the nuclear density (number of nuclei/μm) of individual nerves, and then averaging these values for each nerve. At least 6 peripheral nerves, with total length of at least 1,400 μm, were measured for each genotype. The following combinations had statistically significant differences: *gli-GAL4 +/+UAS-Ras^{V12}* vs. *UAS-Ras^{V12}/+*, p=0.0017; vs. *gli-GAL4/+*, p=0.0001; vs. *gli-GAL4 +/+ UAS-Ras^{+}*, p=0.0075.

This result suggests that hyperactive Ras signalling can, in fact, increase the number of perineurial glial nuclear number. Thus, the *amn^{X8}* and *ine; $NF1^{P2}$* mutants are able to mimic the increased perineurial glial thickness but not perineurial glial nuclear number of larvae expressing *gli-GAL4* and *UAS-Ras^{V12}*. There are several possible explanations for this apparent discrepancy. One possibility is that this apparent discrepancy might reflect the activation by *gli-GAL4* of Ras signalling at a developmental stage in which Ras signalling does not normally occur. Alternatively, *amn, ine* and *NF1* might affect signalling pathways in addition to Ras that prevent the Ras-dependent increase in cell number from occurring. This latter explanation is consistent with other results (described under task one, above) suggesting that *NF1*, but not *Ras*, has dual, opposing effects on perineurial glial thickness. However, further studies will be required to test these various explanations.

KEY RESEARCH ACCOMPLISHMENTS

Perineurial glial nuclear divisions can be genetically separated from perineurial glial growth

Expression of a constitutively active Ras in the peripheral glia can increase the number of perineurial glial nuclei.

REPORTABLE OUTCOMES

1. Manuscript entitled "Ras activity in peripheral glia promotes perineurial glial growth in Drosophila peripheral nerves", by James C. Yager, Alex Rottgers, Michelle C. Wells, Philip E. Caldwell and Michael Stern, was submitted for publication to *J. Neurosci* on May 12, 2003.

2. Abstract entitled " Ras activity in peripheral glia promotes perineurial glial growth in Drosophila peripheral nerves", by James C. Yager, Alexander Rottgers, Michelle C. Wells, Elizabeth L. Carter and Michael Stern, was approved for oral presentation at the NNFF International Consortium meeting, to be held at Aspen, CO, in June, 2003.

3. If we can confirm our observation that expression of $G_{\alpha}s^*$ specifically in peripheral glia causes lethality, then we will be interested in using this system to identify genes downstream of $G_{\alpha}s^*$ by selecting for mutations that suppress this lethal phenotype. Although beyond the scope of this current award, such research would lead us to apply for a patent as well as additional funding from the NIH or other relevant agencies. We have already applied for a provisional patent based on a related observation: that expression of the constitutively active Ras^{V12}, but not Ras^{+} protein, in peripheral glia is lethal at the late pupal stage.

CONCLUSIONS

So far, our research has produced both negative and positive findings. The experiments proposed in task one were hampered by technical and other problems as described above. However, recent work performed from a different grant has led me to believe that the original model on which this task was based was incomplete and should be remodeled. Therefore I recommend that subsequent experiments be modified based on this new model. The experiments proposed in task two failed to support the hypothesis, which was somewhat disappointing. However, preliminary experiments proposed in task three are very exciting. It was anticipated that some negative results would arise from this project, as it is an "idea" award and thus based on hypotheses more speculative than would be found in other projects.

REFERENCES

Yager, J., Richards, S., Hekmat-Scafe, D.S., Hurd, D.D., Sundaresan, V., Caprette, D.R., Saxton, W.M., Carlson, J.R., and Stern, M. (2001). Control of *Drosophila* perineurial glial growth by interacting neurotransmitter-mediated signaling pathways. Proc. Natl. Acad. Sci. U.S.A. *98*, 10445-10450.

Yager, J.C., Rottgers, A., Wells, M.C., Caldwell, P.E. and Stern, M. Ras activity in peripheral glia promotes perineurial glial growth in Drosophila peripheral nerves. *J. Neurosci*, submitted.

APPENDICES

1) Preprint of Yager et al. (submitted to *J. Neurosci*)

2) Abstract of presentation to the NNFF Consortium on NF1 and NF2 (Aspen, CO, June, 2003).

Ras activity in peripheral glia promotes perineurial glial growth in Drosophila peripheral nerves

James C. Yager, Alex Rottgers, Michelle C. Wells, Philip E. Caldwell and Michael Stern[1]

Department of Biochemistry and Cell Biology, Rice University, Houston, TX 77251

Abbreviated title: Ras activity promotes perineurial glial growth

Number of text pages: 27

Number of figures: 6

Number of tables: 0

Number of words in abstract: 240

Number of words in introduction: 500

Number of words in discussion: 1,143

[1]Corresponding author: Michael Stern

Dept. of Biochemistry and Cell Biology MS-140

Rice University

PO Box 1892

Houston, TX 77251-1892

(713) 348-5351

FAX: (713) 348-5154

email: stern@rice.edu

Acknowledgements: We are grateful to Angela Lynn, Vanathi Sundaresan and Gia Fazio for technical assistance; Celeste Berg, Gerry Rubin, Kei Ito, Vanessa Auld, and the Drosophila stock center at Bloomington, IN for fly stocks, Daryl Hurd for kindly supplying transmission electron micrographs of *push* and *ine push* peripheral nerves, and

Mary Ellen Lane for comments on the manuscript. This work was supported by NIH grant NS39984, grant DAMD17-02-1-0651 from the DOD Neurofibromatosis Research Program,and a grant from the National Neurofibromatosis Foundation (to MS).

Key words: Ras; Neurofibromatosis; cell nonautonomy; peripheral nerves; Drosophila; genetic analysis

ABSTRACT

Drosophila peripheral nerves comprise a layer of motor and sensory axons, wrapped by an inner peripheral glia (analogous to the mammalian Schwann cell) and an outer perineurial glia (analogous to the mammalian perineurium). It was previously shown that perineurial glial growth is negatively regulated by a number of genes including *push*, which encodes a large Zn^{2+}-finger-containing protein, *amn*, which encodes a putative neuropeptide, *ine*, which encodes a putative neurotransmitter transporter, and *NF1*, the Drosophila orthologue of the human gene responsible for type 1 Neurofibromatosis. *NF1* encodes Neurofibromin, a Ras-GTP-ase activator protein (Ras-GAP). We show that mutations that reduce Ras activity suppress the increased perineurial glial thickness of the amn^{X8} deletion mutant and the *ine; NF1*^{P2} and *ine push* double mutants. We also show that expression of the constitutively active Ras^{V12} mutation specifically in the peripheral glia is sufficient to confer increased perineurial glial growth. Finally, we show that effects of Ras^{V12} on perineurial glial growth are significantly enhanced by mutations in *push*, but not by mutations in *ine* or *NF1*. We conclude that Ras activity is both necessary and sufficient for increased perineurial glial growth, and that Ras can promote perineurial glial growth cell-nonautonomously. We further suggest that mutations in *NF1* and *ine*, but not mutations in *push*, increase perineurial glial growth by increasing the Ras-GTP to Ras-GDP ratio. Mutations in *push* could act on a pathway parallel to Ras, or increase Ras signalling independently of an effect on [Ras-GTP].

One disease that affects peripheral nerves is type 1 Neurofibromatosis. This autosomal dominant genetic disorder is caused by mutations in *NF1* (reviewed in

Cichowski and Jacks, 2001). The most prominent manifestation in afflicted individuals is the formation of neurofibromas (tumors of the peripheral nerve), which are thought to arise in individuals heterozygous for *NF1* following spontaneous loss of the $NF1^+$ allele within certain cells of the peripheral nerve. However, issues concerning the mechanism of tumor formation remain incompletely understood. For example, neurofibromas contain cells derived from all of the cell types found in peripheral nerves, including Schwann cells, perineurial cells, and fibroblasts. These cell types are not clonally related and it is unlikely that $NF1^+$ is lost simultaneously from each. One explanation for this unexpected property is that *NF1* can act cell-nonautonomously. In this view, the neurofibroma comprises a core of $NF1^-$ cells that cause overproliferation in their heterozygous neighbors via the excessive release of a growth factor. In addition, although Neurofibromin (the protein encoded by *NF1*) exhibits Ras GTPase-activating activity, which negatively regulates Ras, it has been difficult to determine the extent to which hyperactivated Ras causes neurofibroma formation. For example, Ras hyperactivation is observed in only a subpopulation of cells from neurofibromas (Sherman et al., 2000), raising the possibility that other molecular targets of *NF1*, such as adenylate cyclase (The et al., 1997; Guo et al., 1997; Tong et al., 2002), could participate in neurofibroma formation.

Yager et al. (2001) previously reported that mutations in five genes could increase the growth of the outer perineurial glial layer. These five genes include *ine*, which encodes a putative neurotransmitter transporter (Soehnge et al., 1996), *eag*, which encodes a potassium channel (Drysdale et al., 1991; Warmke et al., 1991), *push*, which encodes a large, Zn^{2+}-finger-containing protein (Yager et al., 2001), *amn*, which encodes

a putative neuropeptide related to the pituitary adenylate cyclase activator peptide (PACAP) (Feany and Quinn, 1995), and *NF1*, the Drosophila orthologue of human *NF1* (The et al., 1997). From the growth phenotypes of particular single and double mutants, a model was proposed in which perineurial glial growth is controlled by a growth factor released from peripheral glia. This growth factor release is regulated by two neuron-peripheral glia signalling pathways: one mediated by the Amn neuropeptide acting upstream of *push* and *NF1*, and the second by the substrate neurotransmitter of Ine.

Here we use this genetic system to address a possible cell non-autonomous role of Ras in promoting perineurial glial growth. We find that mutations that reduce Ras activity suppress the increased perineurial glial thickness of amn^{X8}, *ine push* and *ine;* $NF1^{P2}$ mutants. We also find that expression of the constitutively active Ras^{V12} allele, but not Ras^{+}, specifically within peripheral glia increases perineurial glial growth. Thus, Ras activation is both necessary and sufficient to promote perineurial glial growth, and Ras can do so cell nonautonomously. Finally, we provide evidence that *ine* and *NF1* mutations, but not *push* mutations, increase perineurial glial growth by increasing the [Ras-GTP] to [Ras-GDP] ratio.

MATERIALS AND METHODS

Drosophila stocks, mutations and crosses. amn^{X8} is a deletion of the *amn* ORF (Moore et al., 1998) and was supplied by Ulrike Heberlein (University of California, San Francisco); *ine* is a transcript null mutation (Soehnge et al., 1996); $NF1^{P2}$ is a protein null mutation caused by a P element insertion (The et al., 1997) and supplied by Andre

Bernards (Harvard Medical School, Boston, MA); *push* is an early nonsense mutation (Yager et al., 2001); Ras^{12A} and Ras^{e2F} are loss of function mutations that are viable in heteroallelic combination (Zhong, 1995) and supplied by Celeste Berg (University of Washington, Seattle) and Gerry Rubin (University of California, Berkeley), respectively; *gli-GAL4, MZ709* and *MZ317* are three lines that express *GAL4* in peripheral glia (Ito et al., 1995; Auld et al., 1995; Leiserson et al., 2000; Sepp and Auld, 1999) and provided by Vanessa Auld (University of British Columbia, Vancouver, CA) and Kei Ito (National Institute for Basic Biology, Okazaki, Japan), respectively; *UAS-*Ras^{V12} and *UAS-*Ras^{+} express Ras^{V12} and Ras^{+} under the transcriptional control of Gal4 and were provided by the Drosophila stock center (Bloomington, IN).

Standard Drosophila genetics techniques were used to establish the fly stocks and to perform the crosses used in the experiments described. Because Ras^{e2F} is recessive lethal, we obtained Ras^{12A}/Ras^{e2F} larvae by crossing lines heterozygous for each *Ras* mutation and the *TbTM6* third chromosome balancer, and then choosing the non-tubby larvae. Because *push* mutations confer male sterility, we obtained larvae homozygous for *push* by constructing lines heterozygous for *push* and chromosomes carrying a reciprocal translocation between the second chromosome balancer *CyO* and *TbTM6* as described previously (Richards et al., 1996) and then choosing the non-tubby larvae. For all experiments using either *GAL4* or *UAS-Ras* transgenes, the appropriate larvae were obtained following a cross of the *GAL4*-containing fly line to the *UAS-Ras*-containing fly line.

Transmission electron microscopy:

Larvae were grown to the wandering third instar stage in uncrowded half-pint bottles at room temperature (23 degrees). Larvae were collected only during the first and second days after the initial third instar larvae appeared. The dissections, fixations and stainings were performed as previously described (Yager et al., 2001). Perineurial glial thickness was measured from the edge of the nerve to the axon-containing lumen and averaged from eight measurements made 12, 3, 6, 9 o'clock and four positions in between. Measurements were not taken at positions where a perineurial glial nucleus was encountered.

Confocal microscopy: Wandering third instar larvae were grown as described above, filleted, fixed in 5% ρ-formaldehyde in PBS, 0.1% Triton X-100 for 1 hour, treated with RNaseA (400 µg, for 2 hours) and stained with 5 µg propidium iodide for 1 hour. The ventral ganglion and nerves were then removed from the carcass. Nucleii within the peripheral nerves were visualized and digitized by fluorescent laser-scanning confocal microscopy (Zeiss LSM-410). Nerve lengths and nuclei counts were determined following digitization.

RESULTS

Ras activity is required for the increased perineurial glial thickness observed in *amn*^{X8}, *ine push* and *ine; NF1*^{P2} mutants

The previous demonstration that Neurofibromin exhibits Ras-GAP activity and thus negatively regulates Ras activity (Xu et al., 1990; Martin et al., 1990) raised the possibility that the increased perineurial thickness observed in larvae double mutant for

ine and the $NF1^{P2}$ insertion mutant, as well as in amn^{X8} or *ine push* mutant larvae, resulted from elevated Ras activity. If so, then the introduction of loss of function *Ras* mutations would be predicted to suppress the increased perineurial glial thickness of each of the mutants described above. To test this possibility, we performed transmission electron microscopy on cross sections of peripheral nerves of amn^{X8}, *ine push* and *ine;* $NF1^{P2}$ larvae, each heterozygous for the *Ras* loss of function alleles $Ras^{1^{2A}}$ and Ras^{e2F}, and measured perineurial glial thickness. This heteroallelic *Ras* combination was chosen because it was previously shown to reduce Ras activity sufficiently to produce a loss of function phenotype, and yet retain viability (Zhong, 1995). We found that this combination significantly suppressed the increased perineurial glial thickness in amn^{X8}, *ine push* and *ine;* $NF1^{P2}$ (Figure 1). This result demonstrates that Ras activity is required for the increased perineurial glial thickness observed in these mutants.

Expression of the constitutively active Ras^{V12} allele in peripheral glia increases perineurial glial growth

To test the possibility that Ras activation alone is sufficient to promote perineurial glial growth, we expressed the constitutively active Ras^{V12} mutation in otherwise wildtype flies. Ras^{V12} confers constitutive activity by blocking the Ras-GTPase activity, thus locking Ras in the GTP-bound form (Bourne et al., 1991). We predicted that if Ras is the only relevant cellular target of Neurofibromin, then expression of Ras^{V12} should phenocopy the $NF1^{P2}$ mutation and that larvae expressing Ras^{V12} should be indifferent to the presence or absence of $NF1^{P2}$. If, however, *NF1* has cellular targets in addition to Ras, such as adenylate cyclase, that are relevant to perineurial glial growth control, then Ras^{V12} expression should at most incompletely phenocopy *NF1* mutations, and the

phenotype of larvae expressing Ras^{V12} should be different in an $NF1^{P2}$ than in an $NF1^{+}$ background. These experiments were performed in the background of Ras^{+} protein produced from the endogenous *Ras* gene. We anticipated that sufficient overexpression of Ras^{V12} could increase [Ras-GTP] to a level sufficient to generate a dominant phenotype.

Although the *NF1* mutant phenotype is observed in perineurial glia, we chose to express Ras^{V12} in the peripheral glia because a number of recent experiments performed in mice and human cell lines suggested that *NF1* acts in Schwann cells to control peripheral nerve growth (Kluwe et al., 1999; Sherman et al., 2000; Zhu et al., 2002). If *NF1* were to act cell nonautonomously, then *Ras* would be predicted to do so as well. We used the *GAL4/UAS* system (Brand and Perrimon, 1993) to achieve targeted expression of Ras^{V12} within peripheral glia. Three lines of flies bearing *GAL4* insertions with peripheral glial expression: *gli-GAL4*, *MZ709* and *MZ317* (Ito et al., 1995; Leiserson et al., 2000), were used to induce expression in flies bearing *UAS-*Ras^{V12}, which were created in the lab of Denise Montell (Lee et al., 1996).

We found that larvae bearing *gli-GAL4* and *UAS-*Ras^{V12} exhibited an extremely thickened perineurial glia, even in an ine^{+} background. The thickness observed, 2.4 μm, was significantly thicker than the value observed in larvae carrying *gli-GAL4* or *UAS-*Ras^{V12} alone (Figure 2). This increased thickness is not a consequence merely of *Ras* overexpression, because overexpression of Ras^{+}, achieved by crossing *gli-GAL4* and *UAS-*Ras^{+}, did not significantly affect perineurial glial thickness (Figure 2). It therefore appears that increasing the ratio of activated to inactive Ras (increasing [Ras-GTP]), is sufficient to promote perineurial glial growth. These observations also confirm that

activating Ras specifically within peripheral glia can promote perineurial glial growth: Ras can act cell nonautonomously

Regulation of Ras-GTP levels by *NF1* and *ine*

If Neurofibromin exerts its effects on perineurial glial growth entirely by reducing [Ras-GTP], then perineurial glial thickness in larvae expressing *gli-GAL4* and *UAS-Ras^{V12}* should be no thicker in an *NF1^{P2}* mutant than *NF1^{+}* background. In fact, we found that the *NF1^{P2}* mutation significantly reduced perineurial glial thickness in larvae carrying *gli-GAL4* and *UAS-Ras^{V12}* (Figure 2). This unexpected result might indicate that Neurofibromin has an additional activity other than reducing [Ras-GTP]; however this activity would promote, rather than inhibit, perineurial glial growth. Dual, mutually antagonistic roles for *NF1* have previously been observed; in mouse Schwann cells, Nf1 activates a K channel by down-regulating Ras, but inhibits this channel by down regulating [cAMP] (Xu et al., 2002). A similar phenomenon might be occurring in the Drosophila peripheral nerve. Further tests of this possibility are required but are beyond the scope of this manuscript.

Mutations in *ine* enhance perineurial glial thickness in double mutant combination with *NF1* or *push* mutations (Yager et al., 2001). We found that in contrast to this enhancement, the *ine* mutation had no effect on perineurial glial growth in larvae expressing *gli-GAL4* and *UAS-Ras^{V12}* (Figure 2). Thus, it appears that *ine* affects a process that is limiting in *Ras^{+}* but not *Ras^{V12}*-expressing larvae. One possibility is that *ine* regulates Ras signalling and perhaps, like *NF1*, affects [Ras-GTP].

We found that a second line that expresses *GAL4* in peripheral glia, *MZ709*, also increases perineurial glial thickness in the presence of *UAS-Ras^{V12}*, but only in an *ine*

mutant background, not in an *ine*$^{+}$ background (Figure 3). The perineurial glial thickness observed in *ine; MZ709 +/UAS-Ras*^{V12} + is very similar to the value observed in *gli-GAL4/+; UAS-Ras*^{V12}*/+* larvae and is significantly greater than in *ine* mutants expressing either MZ709 or *UAS-Ras*^{V12} alone. We conclude that *MZ709* does not induce *Ras*^{V12} expression as strongly as *gli-GAL4*. In consequence, the ratio of [Ras-GTP] to [Ras-GDP] is less elevated when *UAS-Ras*^{V12} is driven by *MZ709* than by *gli-GAL4*. Thus, the *MZ709/UAS-Ras*^{V12} genotype requires the *ine* mutation to increase Ras signalling to a level required for the perineurial glial growth phenotype to be observed.

A third *GAL4* line that expresses in peripheral glia, *MZ317*, was reported to express less *GAL4* in peripheral glia than *gli-GAL4* or *MZ709* (Leiserson et al., 2000). We found that larvae carrying both *MZ317* and *UAS-Ras*^{V12} failed to exhibit increased perineurial glial thickness even in an *ine* mutant background (Figure 3). We conclude that this *GAL4* line does not induce sufficient *Ras*^{V12} expression to generate an observable phenotype. Thus, with respect to *Ras* expression at least, it appears that an allelic series exists among the peripheral glial drivers: *gli-GAL4* is the strongest driver, whereas *MZ317* is the weakest.

Increased number of perineurial glial nucleii in larvae expressing *Ras*^{V12} in peripheral glia

A second line of evidence that *Ras*^{V12} affects the perineurial glia cell nonautonomously comes from examining the effects of expression of *Ras*^{V12} in peripheral glia on the number of perineurial glial nuclei. The perineurial glia is induced during embryogenesis from mesodermal precursors surrounding the developing peripheral nerve (Edwards et al., 1993). We stained peripheral nerves from filleted third instar larvae with

propidium iodide, which allows the visualization of the peripheral glial and perineurial nuclei by confocal microscopy. Most of the nuclei visualized will be from the perineurial glia, because there are only 6-8 peripheral glial nuclei per nerve (Sepp et al., 2000). We found that the number of perineurial glial nuclei was significantly increased in larvae expressing both *gli-GAL4* and *UAS-Ras^{V12}* (Figure 4) compared to larvae expressing *gli-GAL4* alone, *UAS-Ras^{V12}* alone, or both *gli-GAL4* and *UAS-Ras^{+}*. It is not known if these extra nuclei result from increased nuclear division, increased recruitment of perineurial glial cells from the mesodermal precursors, or reduced apoptosis.

However, the increased glial growth observed in the mutants and double mutants described above does not require any increase in perineurial glial nuclear number. We counted perineurial glial nuclei from *amn^{X8}* and *ine; NF1^{P2}* larvae and found no significant difference compared to wildtype (Figure 4). The ability of *amn^{X8}* and *ine; NF1^{P2}* mutants to mimic the increased perineurial glial thickness but not perineurial glial nuclear number of larvae expressing *gli-GAL4* and *UAS-Ras^{V12}* might reflect the activation by *gli-GAL4* of Ras signalling at a developmental stage in which Ras signalling does not normally occur. Alternatively, *amn, ine* and *NF1* might affect signalling pathways in addition to Ras that prevent the Ras-dependent increase in cell number from occurring. In any case, this result demonstrates in a second way that Ras can affect the perineurial glia cell nonautonomously.

Enhancement of the *Ras^{V12}* phenotype by mutations in *push*

Mutations in *push* enhance the perineurial glial thickness of both *NF1^{P2}* and *ine* mutants (Yager et al., 2001). In contrast to the lack of enhancement of the *NF1^{P2}* or *ine* mutations on larvae carrying *gli-GAL4* and *UAS-Ras^{V12}*, we found that *push* mutations

conferred a significant increase in thickness to larvae expressing Ras^{V12}. In particular, *push* mutants carrying *UAS-*Ras^{V12}, even in the absence of any *GAL4* driver, exhibited a perineurial glial thickness of 3.3 μm, which is about two-fold greater than the value in *push* mutant larvae, or $push^{+}$ larvae carrying UAS-Ras^{V12} (Figure 5). The addition of *gli-GAL4* to the *push; UAS-*Ras^{V12} larvae did not significantly affect perineurial glial thickness (data not shown). We conclude that, unlike *ine* and *NF1*, *push* does not regulate perineurial glial growth by affecting [Ras-GTP]. Instead, *push* might regulate a pathway parallel to Ras, or regulate Ras signalling by a manner distinct from the regulation of [Ras-GTP]. We interpret the ability of *UAS-*Ras^{V12} to enhance *push* mutants even in the absence of any *GAL4* element to reflect leaky (*GAL4*-independent) expression of Ras^{V12} from this transgene. This *GAL4*-independent effect of *UAS-*Ras^{V12} prevents us from concluding that Ras activity within peripheral glia is responsible for the interaction with the *push* mutation, although this possibility would certainly be the simplest. Because we detected a strong *GAL4*-independent phenotype of *UAS-*Ras^{V12} only in a *push* mutant background, but not in an otherwise wildtype, *ine* mutant, or $NF1^{P2}$ mutant background, we suggest that the *push* mutant is hypersensitive to low levels of constitutive Ras signalling.

DISCUSSION

We report the effects of altered Ras activity on perineurial glial growth. First, we have found that a heteroallelic combination of mutations that reduce Ras activity suppresses the increased perineurial glial growth phenotypes of amn^{X8} mutants and *ine push* and *ine; *$NF1^{P2}$ double mutants. Thus, Ras activity is required for the increased

perineurial glial growth observed in these mutants. Second, expression specifically in peripheral glia of the constitutively active Ras^{V12} allele increases perineurial glial growth. This result demonstrates that Ras activation is sufficient to promote perineurial glial growth, and that Ras can act in a cell nonautonomous manner to accomplish this growth promotion. Third, we have found that the increased perineurial glial growth elicited by Ras^{V12} is not further enhanced by the simultaneous presence of either the *ine* or $NF1^{P2}$ mutation. This observation raises the possibility that both *ine* and *NF1* act by regulating [Ras-GTP]. In contrast, we have observed that perineurial glial thickness in *push* mutants is strongly increased in the presence of Ras^{V12}, suggesting that push controls perineurial glial growth by a mechanism distinct from the regulation of [Ras-GTP]. These conclusions are summarized in Figure 6.

Cell nonautonomous effects of Ras activation

The notion that *NF1* acts cell nonautonomously to control growth and proliferation within peripheral nerves is based in part on the cellular heterogeneity of neurofibromas, a property not expected for a tumor caused by loss of a tumor suppressor gene such as *NF1*. Recent evidence from mammalian systems has suggested that *NF1* acts within the Schwann cell component of peripheral nerves to control growth within peripheral nerves (Kluwe et al., 1999; Sherman et al., 2000; Zhu et al., 2002), which suggests that *NF1* controls the proliferation of the non-Schwann cell component of peripheral nerves cell nonautonomously. Our observation that increasing [Ras-GTP] in the peripheral glia (the Drosophila analogue of the Schwann cell) is sufficient to promote perineurial glial growth demonstrates that *Ras* (and by extension, *NF1*) controls growth

within Drosophila peripheral nerves cell nonautonomously as well. Ras activity within peripheral glia might control the production or release of a factor that activates perineurial glial growth.

Neurofibromin negatively regulates perineurial glial growth by inhibiting Ras

Although the ability of Neurofibromin to activate the Ras-GTPase is its most prominent function, Neurofibromin has also been observed to activate adenylate cyclase and thus increase [cAMP] (The et al., 1997; Guo et al., 1997; Tong et al., 2002). This observation raises the possibility that a reduction in [cAMP] contributes to the effect of *NF1* mutations on perineurial glial growth. We observed that a reduction of Ras activity completely suppressed the *ine;* $NF1^{P2}$ glial growth phenotype, and that the increased perineurial glial growth elicited by Ras^{V12} was not further increased by $NF1^{P2}$. In fact, we observed that the $NF1^{P2}$ mutation partially suppressed the increased perineurial glial growth elicited by *gli-GAL4* and *UAS-*Ras^{V12}. Therefore, Ras GTPase-activation is the only activity of Neurofibromin that we can detect that negatively regulates perineurial glial growth. However, Neurofibromin might have additional activities that promote perineurial glial growth.

Regulation of Ras signalling by *ine*

There are two reasons for speculating that *ine*, like *NF1*, regulates perineurial glial growth by regulating Ras signalling and possibly [Ras-GTP]. The first comes from the observation that *ine* mutations significantly enhance the perineurial glial thickness of *NF1* mutants (Yager et al., 2001), to levels similar to those observed in larvae expressing

gli-GAL4 and *UAS-Ras*^{V12}. This observation can be interpreted to suggest that both *ine* and *NF1* mutations increase [Ras-GTP]. Because of partial redundancy, the increase in the single mutants is not sufficient to cause a growth phenotype. However the simultaneous loss of *ine* and *NF1* in the double mutant leads to a larger increase in [Ras-GTP] that becomes sufficient to generate a perineurial glial growth phenotype. The second comes from the observation that the *ine* mutation has no further effect on perineurial glial growth in larvae expressing *gli-Gal4* and *UAS-Ras*^{V12}. This observation suggests that the component of signalling regulated by *ine* is limiting in a Ras^{+} but not Ras^{V12}, background. We speculate that this limiting component might be [Ras-GTP].

Although it appears to be clear how *NF1* negatively regulates [Ras-GTP], it is less immediately obvious how *ine* could do the same. *ine* encodes a member of the Na/Cl-dependent neurotransmitter transporter family (Soehnge et al., 1996). Members of this family generally exert their physiological effects by performing re-uptake of a small molecule neurotransmitter released into the extracellular space, and thus attenuating signalling with this neurotransmitter (Amara and Kuhar, 1993). Yager et al. (2001) suggested that Ine performs re-uptake of a small molecule neurotransmitter released by the motor neuron and acting on the peripheral glia. In this view, the effect of the *ine* mutation on perineurial glial growth would result from defective reuptake, and hence overstimulation of the peripheral glia with neurotransmitter. In several vertebrate and invertebrate systems, it has been found that glia respond to the activity of an underlying neuron or to application of small molecular neurotransmitters such as glutamate with responses dependent upon activation of G-protein coupled receptors. These responses include increases in intracellular [Ca^{s+}] mediated by phospholipase C and IP_3 (Porter and

McCarthy, 1996; Villegas, 1995; Rochon et al., 2001) and the Ras-dependent activation of MAP kinase pathways via the $G_{\alpha}q$-dependent transactivation of the EGF receptor (Luttrell et al., 1999; Peavy et al., 2001). Further studies will be required to determine if hyperactivation of either or both of these processes mediates the effect of *ine* mutations on perineurial glial growth.

A distinct role for *push* in the control of perineurial glial growth

Mutations in *push* strongly enhance the perineurial glial growth phenotype of larvae expressing even low levels of Ras^{V12}. In particular, push mutant larvae, but not wildtype or *ine* or $NF1^{P2}$ mutant larvae, exhibit significantly increased perineurial glial thickness in the presence of *UAS-*Ras^{V12}, despite the absence of any *GAL4* driver. This result suggests that *push* mutant larvae are hypersensitive to the low level of *GAL4*-independent Ras^{V12} expression, and thus to slight elevations of [Ras-GTP]. This hypersensitivity could explain the observation that *ine* and *NF1* mutations, which we have suggested might also slightly elevate [Ras-GTP], each confer a perineurial growth phenotype in a *push* mutant, but not wildtype, background (Yager et al., 2002). These results also strongly suggest that *push*, unlike *ine* and *NF1*, does not regulate [Ras-GTP]. Rather, Push could regulate a pathway parallel to Ras or regulate Ras signalling by a mechanism distinct from regulation of [Ras-GTP]. Ras signalling is known to be regulated by [cAMP] (Cook and McCormick, 1993), through mechanisms such as palmitoylation, which regulates intracellular localization of Ras (Hancock et al., 1990), by Ras signalling scaffolding proteins such as 14-3-3 and Kinase suppressor of Ras (Therrien et al., 1995; Freed et al., 1994) which regulate the formation of

macromolecular complexes containing Ras and its downstream effectors such as Raf, and by Ras-dependent negative feedback (Spencer et al., 1998).

REFERENCES

Amara, S.G., and Kuhar, M.J. (1993). Neurotransmitter transporters: recent progress. Annu. Rev. Neurosci. *16*, 73-93.

Auld, V.J., Fetter, R.D., Broadie, K., Goodman, C.S. (1995). Gliotactin, a novel transmembrane protein on peripheral glia, is required to form the blood-nerve barrier in Drosophila. Cell *81*, 757--767.

Bourne, H.R., Sanders, D.A., and McCormick, F. (1991). The GTPase superfamily: conserved structure and molecular mechanism. Nature *349*, 117-127.

Brand, A., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401-415.

Cichowski, K., and Jacks, T. (2001). NF1 tumor suppressor gene function: Narrowing the GAP. Cell, *104*, 593-604.

Cook, S.J., and McCormick, F. (1993). Inhibition by cAMP of Ras-dependent activation of Raf. Science *262*, 1069-1072.

Drysdale, R.A., Warmke, J., Kreber, R., and Ganetzky, B. (1991). Molecular characterization of eag: a gene affecting potassium channels in Drosophila melanogaster. Genetics *127*, 497-505.

Edwards, J.S., Swales, L.S., and Bate, M. (1993). The differentiation between neuroglia and connective tissue sheath in insect ganglia revisited: The neural lamella and perineurial sheath cells are absent in a mesodermless mutant of Drosophila. J. comp. Neurol. *333*, 301-308.

Feany, M.B., and Quinn, W.G. (1995). A neuropeptide gene defined by the Drosophila memory mutant amnesiac. Science *26*, 869-873.

Freed, E., Symons, M., Macdonald, S.G., McCormick, F., and Ruggieri, R. Binding of 14-3-3 proteins to the protein kinase Raf and effects on its activation. Science *265*, 1713-1716.

Guo, H.F., The, I., Hannan, F., Bernards, A., and Zhong, Y. (1997). Requirement of Drosophila NF1 for activation of adenylyl cyclase by PACAP38-like neuropeptides. Science *276*, 795-798.

Hancock, J.F., Paterson, H., and Marshall, C.J. (1990). A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane. Cell *63*,133-139.

Ito, K., Urban, J., and Technau, G.M. (1995). Distribution, classification, and development of Drosophila glial cells in late embryonic ventral nerve cord. Rouxs Archive Dev. Biol. *204*, 284–307.

Kluwe, L., Friedrich, R., and Mautner, V.F. (1999). Loss of NF1 allele in Schwann cells but not in fibroblasts derived from an NF1-associated neurofibroma. Genes Chromosomes Cancer *24*, 283-285.

Lee, T., Feig, L., and Montell, D.J. (1996). Two distinct roles for Ras in a developmentally regulated cell migration. Development *122*, 409-418.

Leiserson, W.M., Harkins, E.W., and Keshishian, H. (2000). Fray, a Drosophila Serine/Threonine Kinase Homologous to Mammalian PASK, Is Required for Axonal Ensheathment. Neuron *28*, 793-806.

Luttrell, L.M., Daaka, Y., and Lefkowitz, R.J. (1999). Regulation of tyrosine kinase cascades by G-protein-coupled receptors. Curr. Opin. Cell. Biol. *11*, 177-183.

Martin, G.A., Viskochil, D., Bollag, G., McCabe, P.C., Crosier, W.J., Haubruck, H., Conroy, L., Clark, R., O'Connell, P., and Cawthon, R.M. (1990). The GAP-related Domain of the Neurofibromatosis Type Gene Product Interacts with Ras p21. Cell *63*, 843–849.

Peavy, R.D., Chang, M.S.S., Sanders-Bush, E., and Conn, P.J. (2001). Metabotropic glutamate receptor 5-induced phosphorylation of extracellular signal-regulated kinase in astrocytes depends on transactivation of the epidermal growth factor receptor. J. Neurosci. *21*, 9619-9628.

Porter, J.T., and McCarthy, K.D. (1996). Hippocampal Astrocytes In Situ Respond to Glutamate Released from Synaptic Terminals. J. Neurosci. *16*, 5073-5081.

Richards, S., Hillman, T., and Stern, M. (1996). Mutations in the Drosophila pushover gene confer increased neuronal excitability and spontaneous synaptic vesicle fusion. Genetics *142*, 1215-1223.

Rochon, D., Rousse, I., and Robitaille, R. (2001). Synapse-glia interactions at the mammalian neuromuscular junction. J. Neurosci. *21*, 3819-3829.

Sepp, K.J., Auld, V.J. (1999). Conversion of lacZ enhancer trap lines to GAL4 lines using targeted transposition in Drosophila melanogaster. Genetics *151*, 1093--1101.

Sepp, K.J.,Schulte, J., and Auld, V.J. (2000). Developmental dynamics of peripheral glia in Drosophila melanogaster. Glia *30*, 122-133.

Sherman, L.S., Atit, R., Rosenbaum, T., Cox, A.D., and Ratner, N. (2000). Single cell Ras-GTP analysis reveals altered Ras activity in a subpopulation of neurofibroma Schwann cells but not fibroblasts. J Biol Chem *275*, 30740-5.

Soehnge, H., Huang, X., Becker, M., Whitley, P., Conover, D., and Stern, M. (1996) A neurotransmitter transporter encoded by the Drosophila *inebriated* gene. Proc. Natl. Acad. Sci. U.S.A. *93*, 13262-13267.

Spencer, S.A., Powell, P.A., Miller, D.T., Cagan, R.L. (1998). Regulation of EGF receptor signaling establishes pattern across the developing Drosophila retina. Development *125*, 4777-4790.

The, I., Hannigan, G.E., Cowley, G.S., Reginald, S., Zhong, Y., Gusella, J.F., Hariharan, I.K., and Bernards, A. (1997) Rescue of a Drosophila NF1 mutant phenotype by protein kinase A. Science *276*, 791-794.

Therrien, M., Chang, H.C., Solomon, N.M., Karim, F.D., Wassarman, D.A., and Rubin, G.M. (1995). KSR, a novel protein kinase required for RAS signal transduction. Cell *83*, 879—888.

Tong, J., Hannan, F., Zhu, Y., Bernards, A., and Zhong, Y. (2002). Neurofibromin regulates G protein-stimulated adenylyl cyclase activity. Nat. Neurosci. *5*, 95-96.

Villegas, J. (1995). Learning from the axon-Schwann cell relationships of the giant nerve fiber of the squid, in Neuron-Glia interactions during phylogeny: II plasticity and regeneration. (Vernadakis, A., and Roots, B., eds.) pp. 95-127, Humana Press.

Warmke, J.W., Drysdale, R.A., and Ganetzky, B. (1991). A distinct potassium channel polypeptide encoded by the Drosophila eag locus. Science *252* 1560-1562.

Xu, G.F., Lin, B., Tanaka, K., Dunn, D., Wood, D., Gesteland, R., White, R., Weiss, R., and Tamanoi, F. (1990). The catalytic domain of the neurofibromatosis type 1 gene stimulates ras GTPase and complements ira mutants of S. cerevisiae. Cell *63*, 835-841.

Xu, Y., Chiamvimonvat, N., Vazquez, A.E., Akunuru, S., Ratner, N., and Yamoah, E.N. (2002). Gene-targeted deletion of neurofibromin enhances the expression of a transient outward K+ current in Schwann cells: a protein kinase A-mediated mechanism. J. Neurosci. *22*, 9194-9202.

Yager, J., Richards, S., Hekmat-Scafe, D.S., Hurd, D.D., Sundaresan, V., Caprette, D.R., Saxton, W.M., Carlson, J.R., and Stern, M. (2001). Control of *Drosophila* perineurial glial growth by interacting neurotransmitter-mediated signaling pathways. Proc. Natl. Acad. Sci. U.S.A. *98*, 10445-10450.

Zhong, Y. (1995). Mediation of PACAP-like neuropeptide transmission by coactivation of Ras/Raf and cAMP signal transduction pathways in Drosophila. Nature *375*, :588—592.

Zhu, Y., Ghosh, P., Charnay, P., Burns, D.K., and Parada, L.F. (2002). Neurofibromas in NF1: Schwann cell origin and role of tumor environment. Science *296*, 920-922.

Figure 1: Suppression of the increased perineurial glial thickness of amn^{X8}, *ine push*, and *ine;* $NF1^{P2}$ mutants by reduction in Ras activity. A. Transmission electron micrographs of cross sections of typical peripheral nerves from *ine push* and *ine push;* Ras^{12A}/Ras^{e2F}. B. Means and SEMs of perineurial glial thickness from the indicated genotypes. [a]From data published in Yager et al. (2001). The following pairwise combinations had statistically significant differences (two-tailed unpaired t-test): for amn^{X8} (n=24) vs. amn^{X8}; Ras^{12A}/Ras^{e2F} (n=20), p=0.015; for *ine push* (n=14) vs. *ine push;* Ras^{12A}/Ras^{e2F}(n=25), p=0.017; for *ine;* $NF1^{P2}$(n=12) vs, *ine;* Ras^{12A} $NF1^{P2}/Ras^{e2F}$ $NF1^{P2}$ (n=24), p<0.0001.

Figure 2: Effects on perineurial glial growth of expression of constitutively active *Ras* in peripheral glia. A. Transmission electron micrographs of cross sections of typical peripheral nerves from *gli*-GAL4 +/+ $UAS\text{-}Ras^{+}$, *gli-GAL4/+;* $UAS\text{-}Ras^{V12}$/+, and $UAS\text{-}Ras^{V12}$/+ larvae. B. Means and SEMs of perineurial glial thickness from the indicated genotypes. The following pairwise combinations had statistically significant differences (two-tailed unpaired t-test): *gli-GAL4/+;* $UAS\text{-}Ras^{V12}$/+ (n=72) vs. *gli-GAL4/+*, p<0.0001 (n=21); vs. $UAS\text{-}Ras^{V12}$/+, p<0.0001 (n=49); vs. *gli*-GAL4 +/+ $UAS\text{-}Ras^{+}$, p<0.0001 (n=68); and vs. *gli-GAL4/+;* $UAS\text{-}Ras^{V12}$ $NF1^{P2}$/+ $NF1^{P2}$, p=0.0002 (n-41). The pairwise combination *gli-GAL4/+;* $UAS\text{-}Ras^{V12}$ vs. *ine gli-GAL4/ine +;* $UAS\text{-}Ras^{V12}$/+ (n=36) was not significantly different (p=0.49).

Figure 3: Effects on perineurial glial growth of Ras^{V12} expression induced by different peripheral glial *GAL4* elements. A. Transmission electron micrographs of cross-sections

of typical peripheral nerves from larvae of the indicated genotypes. B. Means and SEMs of perineurial glial thickness from the indicated genotypes. The following pairwise combinations had statistically significant differences (two-tailed unpaired t-test): *ine; MZ709 +/+ UAS-Ras^{V12}* (n=27) vs. *ine; UAS-Ras^{V12}/+* (n=27), p=0.0002; vs *ine; MZ709/+* (n=29), p<0.0001; vs. *MZ709 +/+ UAS-Ras^{V12}* (n=23), p=0.0032. The pairwise combination *ine MZ317/ine +; UAS-Ras^{V12}/+* (n=33) *vs. ine; UAS-Ras^{V12}/+* was not significantly different (p=0.725).

Figure 4: Increased number of perineurial glial nuclei in larvae carrying *gli-GAL4* and *UAS-Ras^{V12}*. A. Confocal fluorescent images of peripheral nerves from larvae carrying *gli-GAL4* and *UAS-Ras^{V12}* (left panel) and *gli-GAL4* and *UAS-Ras^{+}* (right panel). The large, nucleus-rich structure on the right of the right panel is the ventral ganglion. B. Means and SEMs of perineurial glial nuclear density from larvae of the following genotypes: a) *K33* (wildtype control for *ine; NF1^{P2}*), b) *ine; NF1^{P2}*, c) wildtype control for *amn^{X8}*, d) *amn^{X8}*, e) *UAS-Ras^{V12}/+*, f) *gli-GAL4/+*, g) *gli-GAL4 +/+ UAS-Ras^{+}*, h) *gli-GAL4 +/+UAS-Ras^{V12}*. Means were calculated by measuring the nuclear density (number of nuclei/μm) of individual nerves, and then averaging these values for each nerve. At least 6 peripheral nerves, with total length of at least 1,400 μm, were measured for each genotype. The following combinations had statistically significant differences: *gli-GAL4 +/+UAS-Ras^{V12}* vs. *UAS-Ras^{V12}/+*, p=0.0017; vs. *gli-GAL4/+*, p=0.0001; vs. *gli-GAL4 +/+ UAS-Ras^{+}*, p=0.0075.

Figure 5: Synergistic enhancement of *push* mutations by low-level expression of Ras^{V12}. A. Transmission electron micrographs of cross sections of typical peripheral nerves from *push* mutant and *push; UAS-*Ras^{V12}/+ larvae. B. Means and SEMs of perineurial glial thickness from the indicated genotypes. [a]From data published in Yager et al. (2001). The following pairwise combinations had statistically significant differences (two-tailed unpaired t-test): *push; UAS-*Ras^{V12}/+ (n=24) vs. *push* (n=20), $p<0.0001$; and vs. *UAS-*Ras^{V12}/+ (n=40), $p<0.0001$.

Figure 6: Model for the control of perineurial glial growth by Ras. Ras-activated production or release of a growth factor from the peripheral glia promotes perineurial glial growth. [Ras-GTP] levels are inhibited by Amn released from the motor neuron acting through Neurofibromin (Guo et al., 1997; Yager et al., 2001) and activated by the substrate neurotransmitter of Ine, called NT, released from the motor neuron. These neurotransmitters could be released from the motor nerve terminal, or along the entire length of the axon, as indicated. Ine and the Eag K^+ channel (acting in the motor neuron) attenuate the effects of NT by performing NT re-uptake (for Ine) or reducing NT release by reducing neuronal excitability (for Eag). Push, also suggested to be a downstream effector of Amn (J. Kramer and R.S. Hawley, personal communication, cited in Yager et al. [2001]), negatively regulates growth factor production or release by a pathway parallel to Ras, or by regulation of Ras signalling in a manner distinct from the regulation of [Ras-GTP].

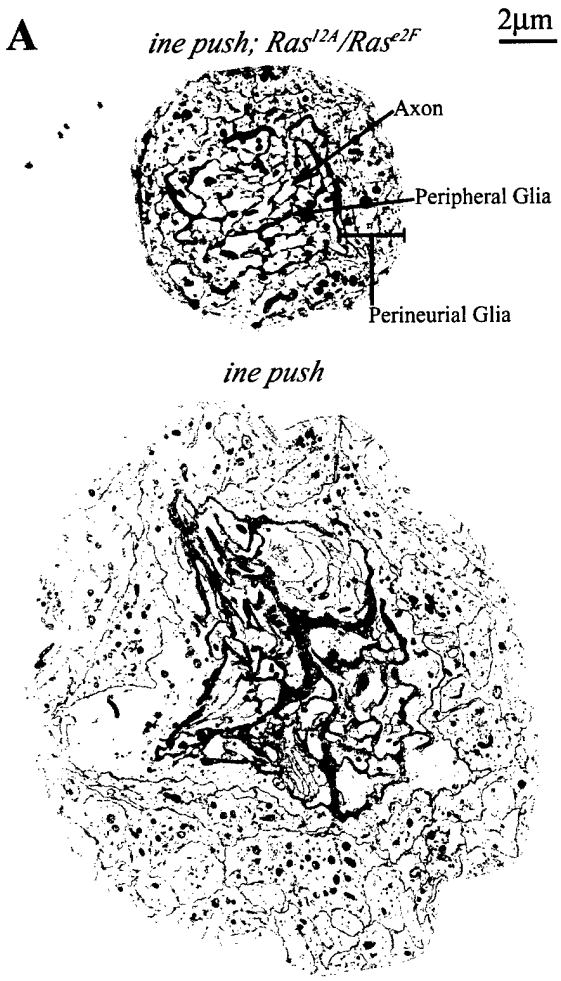

FIGURE 1
Yager 2003

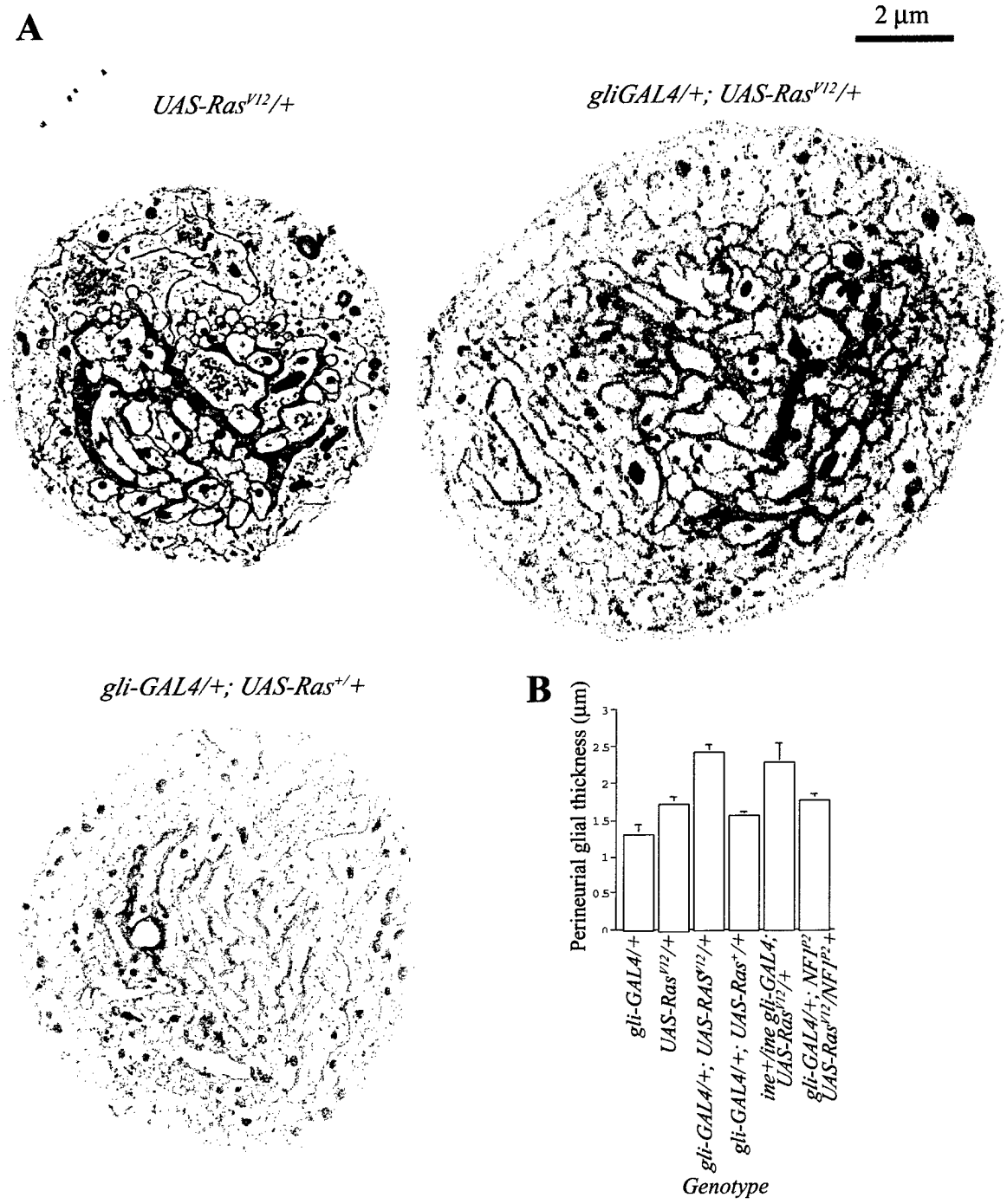

Figure 2
Yager 2003

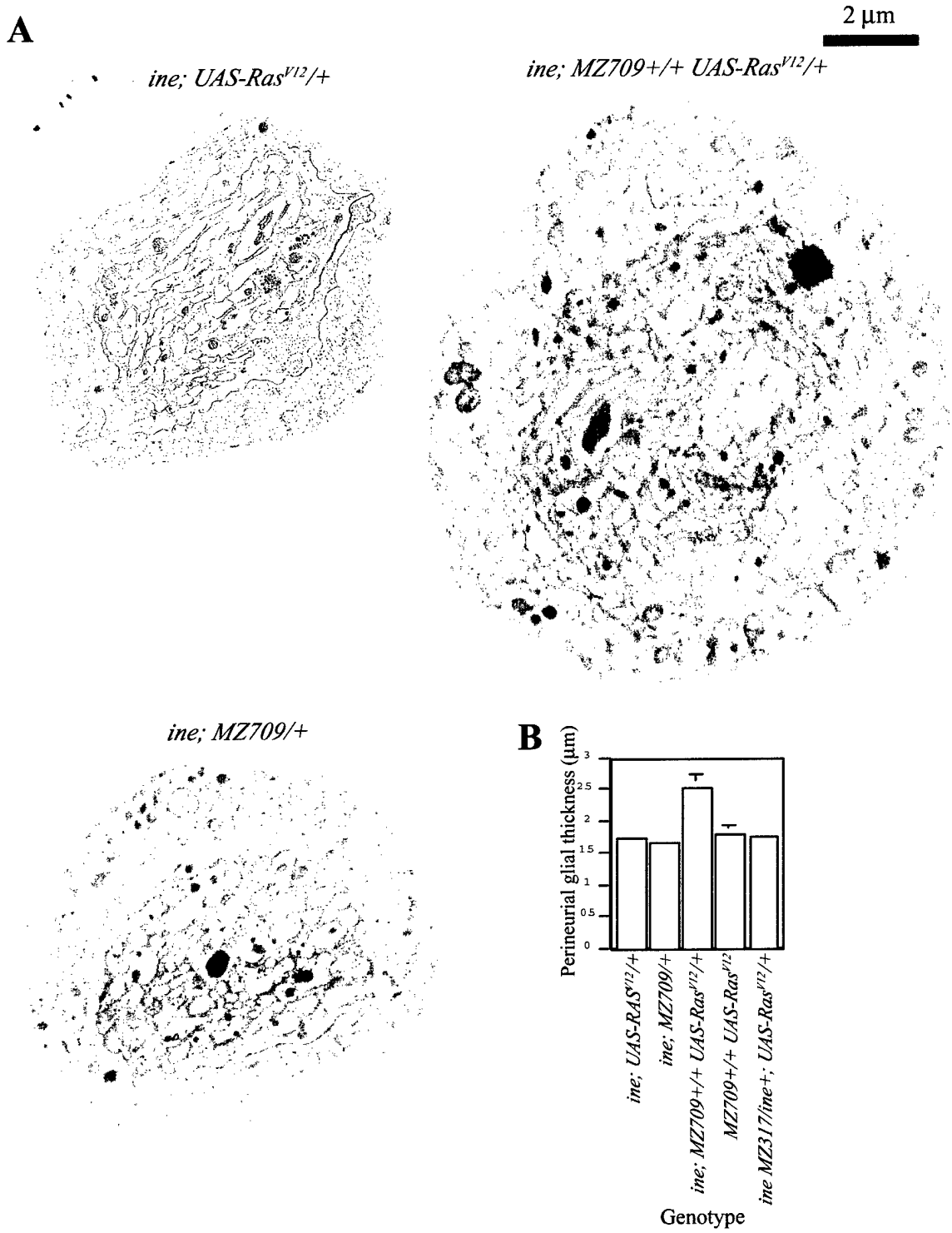

Figure 3
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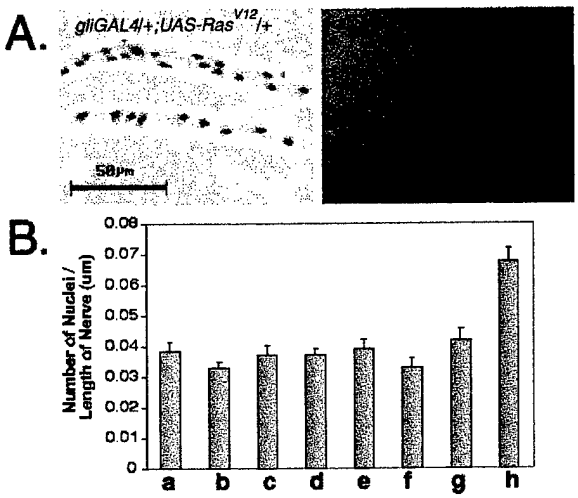

Figure 4
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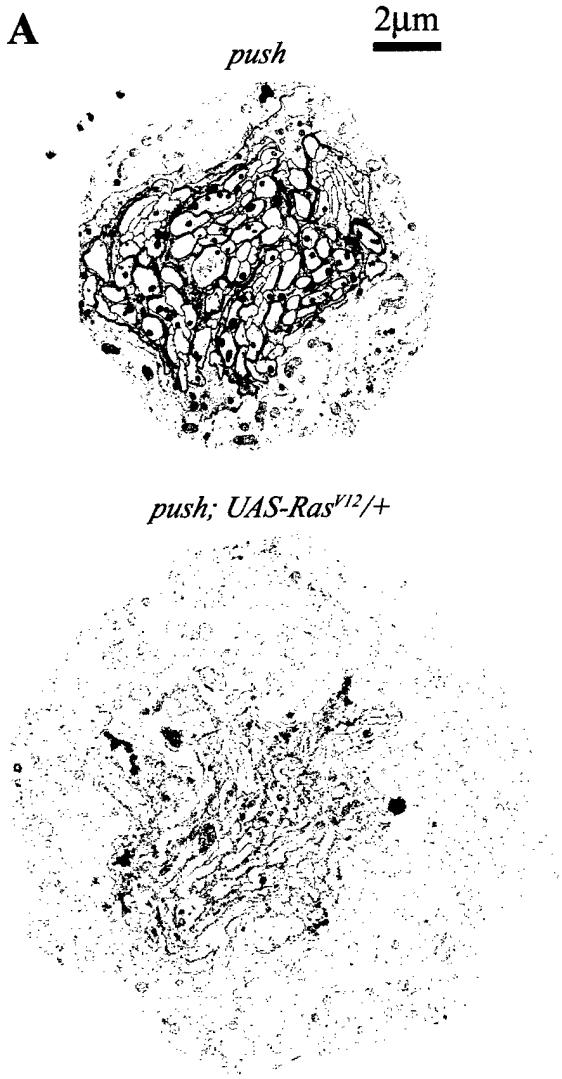

Figure 5
Yager 2003

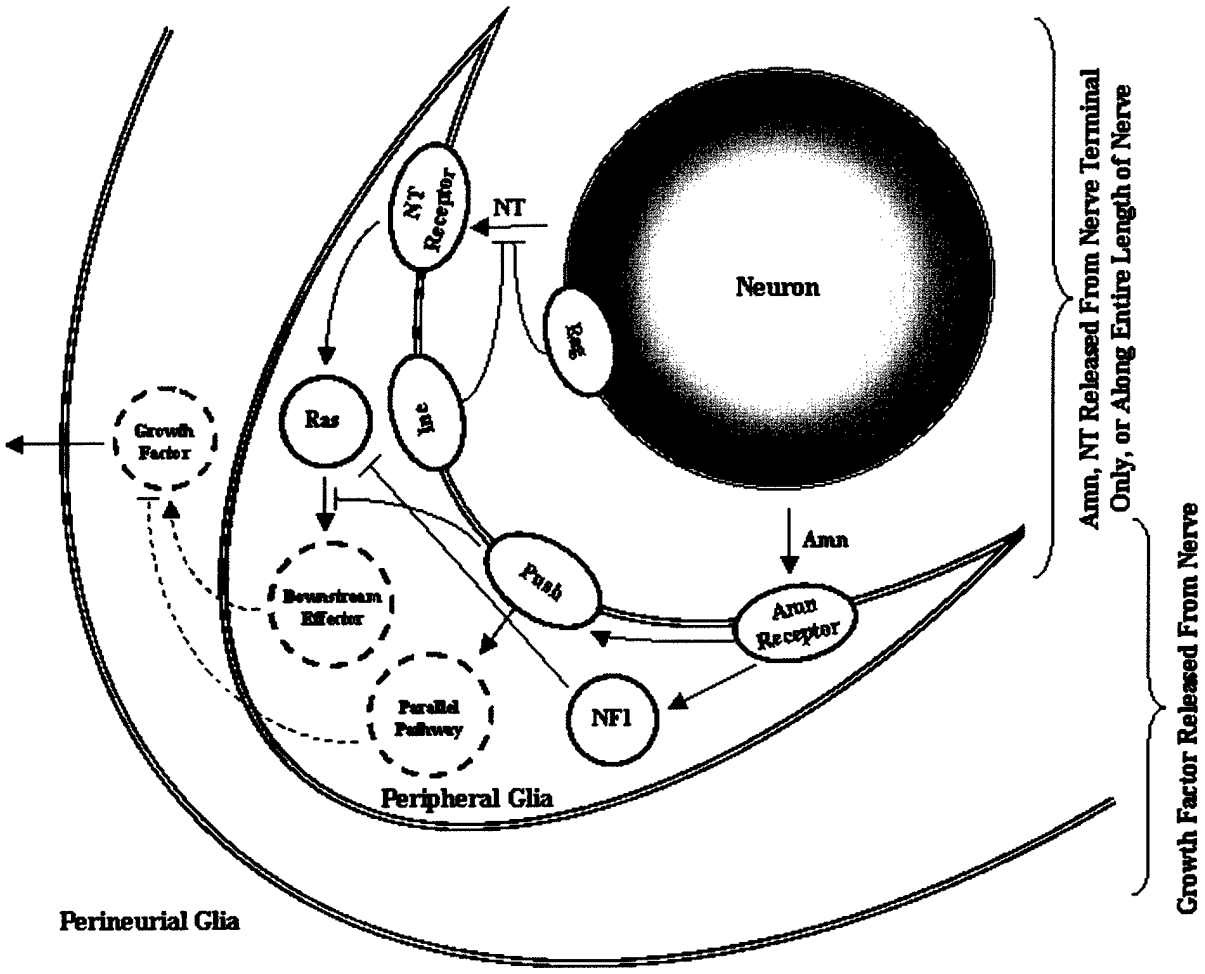

Figure 6
Yager 2003

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ABSTRACT

TITLE: Ras activity in peripheral glia promotes perineurial glial growth in Drosophila peripheral nerves

James C. Yager, Alexander Rottgers, Michelle C. Wells, Elizabeth L. Carter and Michael Stern

Position of presenting author: PI

Affiliation: Dept. of Biochemistry and Cell Biology, Rice University
Address: Dept. of Biochemistry MS-140, Rice University, PO Box 1892, Houston, TX 77251.
Tel: (713) 348-5351 Fax: (713) 348-5154
Email: stern@bioc.rice.edu

Drosophila peripheral nerves comprise a layer of motor and sensory axons, wrapped by an inner peripheral glia (analogous to the mammalian Schwann cell) and an outer perineurial glia (analogous to the mammalian perineurium). It was previously shown that perineurial glial growth in third instar Drosophila larvae is negatively regulated by a number of genes including *push*, which encodes a large Zn^{2+}-finger-containing protein, *amn*, which encodes a putative neuropeptide, *ine*, which encodes a putative neurotransmitter transporter, and *NF1*. We show that mutations that reduce Ras activity suppress the increased perineurial glial thickness of the amn^{X8} deletion mutant and the *ine; NF1*^{P2} and *ine push* double mutants. In contrast, expression of the constitutively active Ras^{V12} mutation specifically in the peripheral glia is sufficient to confer increased perineurial glial growth. We also show that the effect on perineurial glial growth of Ras^{V12} is significantly enhanced by mutations in *push* but not by mutations in *ine* or *NF1*. The *push* mutant, but not the *ine* or *NF1* mutants, also exhibits hypersensitivity to low levels of Ras^{V12} expression. We conclude that Ras activity is both necessary and sufficient for increased perineurial glial growth, and that Ras can promote perineurial glial growth cell-nonautonomously. We further suggest that mutations in *NF1* and *ine*, but not *push*, increase perineurial glial growth by increasing [Ras-GTP]. Mutations in *push* could act on a pathway parallel to Ras, or increase Ras signalling independently of an effect on [Ras-GTP]. Cell nonautonomous effects of Ras activity could be responsible for the cellular heterogeneity of neurofibromas.